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Mushroom Bodies, Ca^{2+} Oscillations, and the Memory Gene *amnesiac*

Minireview

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The memory of odors in *Drosophila* is mediated by mushroom body neurons. Memory is formed, in part, by a modulation of the physiology of these neurons brought about by neuropeptides that are encoded by the *amnesiac* gene and released from peptidergic neurons that innervate mushroom body neurons. Slow and spontaneous oscillations of calcium levels are elevated in the mushroom body neurons of *amnesiac* mutants and may contribute to memory consolidation.

Three big questions recur in much of the literature on the mechanisms of learning and memory. The first is, which of the many neural structures in the brain, and which neurons, have dominion over the formation and storage of memories? The answers for this question not only identify the brain structures responsible for memory, but they are necessary to formulate properly and attack the second and third big questions. The second one is, what physiological and biochemical properties distinguish the neurons responsible for memory, and how do these properties change in response to the formation and storage of memories? The third question concerns how information gets in and around the brain to the neurons that help form memory. That is, how is sensory information conveyed to the relevant neurons? Two recent papers, one published last November in *Cell* by Scott Waddell, Chip Quinn, and colleagues (Waddell et al., 2000), and another published in this issue of *Neuron* by Philippe Rosay, Kim Kaiser, and colleagues (Rosay et al., 2001), offer big steps toward answering these questions in *Drosophila*. Both papers focus on the role of mushroom body neurons in learning about odors and on one mutant defective in odor learning and memory, named *amnesiac* (*amn*).

The Case for Mushroom Bodies

There is now convincing evidence to embrace the hypothesis that mushroom body neurons are mediators of odor learning (see recent reviews: Heisenberg, 1998; Roman and Davis, 2001). Some of this evidence dates from studies conducted in the 19th century. But the last decade of research with *Drosophila* has been especially informative, beginning with the observation that the product of the learning gene *dunce* is quite specifically expressed within these neurons (Nighorn et al., 1991). Nonetheless, complex behaviors including learning emerge from the parallel processing of information by the brain. It seems unlikely that mushroom bodies do it all; other regions of the insect brain probably process and help to form and store odor memories.

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Mushroom bodies are paired structures in the brain formed from approximately 2,500 cells in each hemisphere. Figure 1 illustrates the mushroom body and associated neural structures from one hemisphere of the fly's brain. The mushroom body cells (MBC) reside in the dorsal and posterior cortex of the brain. They extend axons as a bundle through a structure named the peduncle (P) toward the anterior margin of the brain, where the axons turn either dorsally or medially. For some mushroom body neurons, the axons divide from the peduncle into two branches; one branch extends in a dorsal direction and the other in a medial direction. Mushroom body neurons with this architecture are of two types (Crittenden et al., 1998; Lee et al., 1999). Those called α/β neurons extend their axon branches into distinct neuropil regions named the α and β lobes (blue in Figure 1). The second type is the α'/β' neurons, which extend axon branches into the α' and β' lobes (gray in the Figure). These lobes are situated in parallel with the α and β lobes. The third class of mushroom body neurons has an unbranched axon. The γ neurons extend their axon medially after emergence from the peduncle into the γ lobe (deep blue).

Mushroom body neurons receive sensory information about odors, carried from the olfactory neurons on the antennae to glomeruli of the antennal lobe (AL) and, in turn, to the mushroom body dendrites via relay neurons in the antennal lobe and their axons in the antennal cerebral tract (ACT). The axons of relay neurons synapse with the dendrites of mushroom body neurons in a neuropil region named the calyx (C). In addition, the ACT continues beyond the calyx to transmit information about odors to another neuropil region named the lateral protocerebrum (LPC). Virtually nothing is known about the function of the LPC, but this brain region along with the mushroom bodies and the antennal lobes comprise the major primary targets for olfactory information in the insect brain. Thus, learning about odors by insects is predicted strictly on the basis of anatomy to take place along the neural pathway that includes the antennal lobes, mushroom bodies, and lateral protocerebrum.

The pleasing picture that has emerged from the study of expression patterns of genes known to be involved in *Drosophila* olfactory learning is that they are, in general, expressed preferentially in the mushroom body neurons (Roman and Davis, 2001)! This is true for the genes *dunce* (*dnc*, cAMP phosphodiesterase), *rutabaga* (*rut*, adenylyl cyclase), *DCO* (protein kinase A catalytic subunit), the protein kinase A regulatory subunits (RI and RII), *leonardo* (*leo*, 14-3-3), *Volado* (*Vol*, α-integrin), and *fasiclin II* (*fasII*) (Figure 2; see also Cheng et al., 2001). Zars et al. (2000) have also recently reported that restoring normal *rut* function in the mushroom bodies of *rut* mutant animals restores learning. For these and other reasons (Roman and Davis, 2001), the biochemical and physiological models for learning in *Drosophila*, so far, have revolved around intrinsic mushroom body neurons and their place in the olfactory pathway (Figure 2).

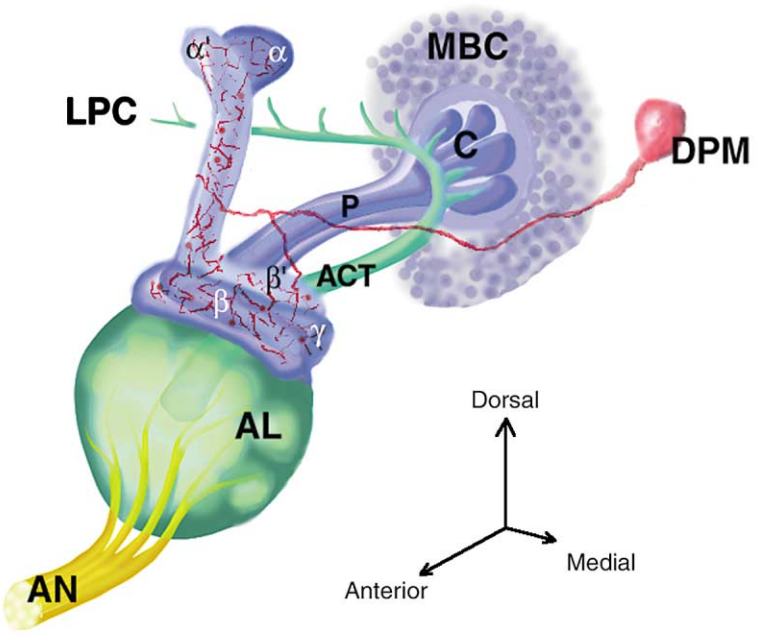

Figure 1. Mushroom Bodies of *Drosophila* and Associated Neural Structures

A mushroom body in the right brain hemisphere of a fly is illustrated using a viewpoint from the fly's front and left. The mushroom body is shown in shades of blue and gray. See text for abbreviations. Neural structures involved in transmitting and processing primary olfactory information are illustrated in shades of yellow and green. One member of the dorsally paired medial neurons (DPM) whose cell body resides medially to its ipsilateral mushroom body is illustrated in red. The DPM axon extends in an anterior direction toward the mushroom body lobes where it divides, sending one branch to broadly innervate the α/α' lobes and another to broadly innervate the $\beta/\beta'/\gamma$ lobes. Figure adapted from Armstrong et al. (1998).

AMN as a Modulator of Mushroom Body Neurons

This is where the elegant story by Quinn and colleagues intersects the mushroom body hypothesis so beautifully, at first glance, in a seemingly tangential way. The learning mutant *amn* was isolated more than 20 years ago (Quinn et al., 1979) but has not been studied relative to the gene's expression pattern until recently. Quinn and colleagues studied this issue using antisera prepared against the predicted *amn* gene product and with *amn* reporter genes but failed to find preferential expression of AMN in mushroom body neurons like other known learning genes (Figure 2). Nor did they find preferential expression in other structures along the olfactory pathway such as the antennal lobes or lateral protocerebrum (Figure 1). Rather, they discovered that *amn* is quite specifically expressed in a pair of neurons called the dorsal paired medial (DPM) neurons that are situated medially to the mushroom bodies (Figures 1 and 2). Intriguingly, these neurons project axons to and broadly innervate the ipsilateral mushroom body lobes.

The sequence of the *amn* gene predicts a protein with features of a preproneuropeptide with some amino acid sequence similarity to pituitary-adenylyl cyclase-activating peptide, also called PACAP (Feany and Quinn, 1995; Moore et al., 1998). Behaviorally, *amn* mutants show a reduction in memory immediately after training (often referred to as learning) as well at later times (DeZazzo et al., 1999; Waddell et al., 2000). This phenotype is similar, both qualitatively and quantitatively (DeZazzo et al., 1999), to other learning mutants such as *rut*. Given that neuropeptides are often coreleased with classical neurotransmitters but generally have slower and longer-lasting postsynaptic effects, the discovery of AMN axon terminals in the mushroom body lobes presents the attractive hypothesis that AMN peptides may be released upon mushroom body axons or their terminals to produce relatively long-lasting, physiological changes (Figure 2). Indeed, it has been shown that application of vertebrate PACAP to the *Drosophila* neuromuscular junction induces a rather slow and long-lasting (minutes) increase in voltage-dependent potassium channel activity (Zhong, 1995).

But is this intersection with mushroom bodies coincidental, or do the DPM neurons really play a role in olfactory learning and memory? The Quinn group employed a powerful and novel reagent constructed by Toshihiro Kitamoto (Kitamoto, 2001) to demonstrate DPM importance to learning. This reagent is a transgene that encodes a dominant and temperature-sensitive form of the *Shibire* gene product (dynamin), which is normally required for neurotransmitter vesicle endocytosis. At the restrictive temperature, endocytosis is blocked, new vesicle formation is dramatically attenuated, and neurotransmission stops. Using promoters to drive the expression of the transgene in the DPM neurons, the researchers tested whether switching off (with elevated temperature) neurotransmission from the DPM neurons would impair memory. Surprisingly, this treatment had no effect upon memory immediately after training. However, continuous heat treatment from the time of training to testing blocked completely 1 hr memory. This strongly suggested the independence of DPM neurons for learning and immediate memory, but an involvement in the storage or retrieval of memories at 1 hr.

To close the circle and connect these observations with the *amn* gene and mutants, they then selectively expressed in *amn* mutants a wild-type *amn* transgene in the DPM neurons. This expression rescued the *amn* phenotype, thus suggesting that expression within the DPM neurons is sufficient for normal learning and memory. Since the promoters used to drive the expression are active both during development and in the adult animal, it is possible that the *amn* defect of memory immediately after training reflects a developmental requirement for the gene products. The role of *amn* in later memory may be dependent solely upon DPM neuron activity in the adult.

The overall picture that emerges is that the *amn* gene

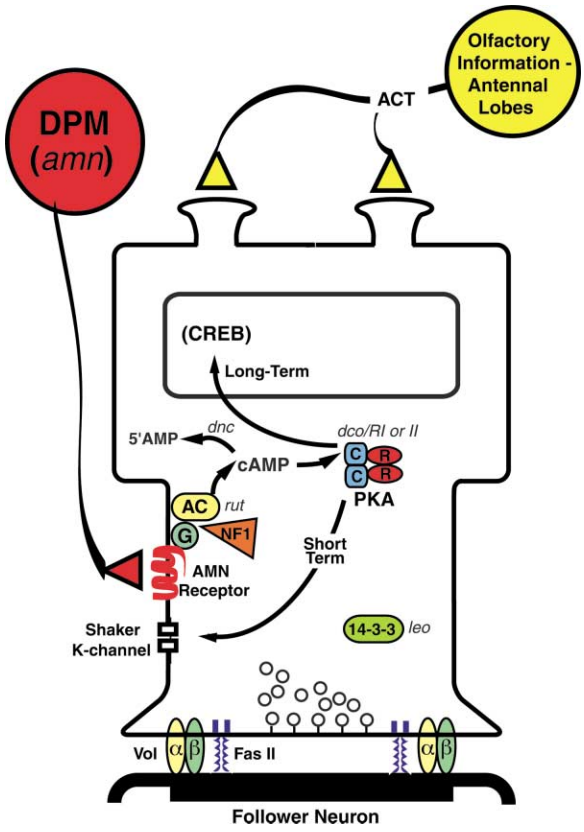

Figure 2. Modulation of the Biochemistry of a Mushroom Body Neuron

An illustration of a mushroom body neuron with many of the known molecules involved in olfactory learning. The dendrites of the mushroom body neurons receive olfactory information from the antennal lobes via the ACT. The *rut*-encoded adenylyl cyclase is expressed principally in the axons and axon terminals of mushroom body neurons and is known to be linked to G protein-coupled receptors. The DPM neuron axons also are hypothesized to synapse on mushroom body axons or their terminals to release modulatory neuropeptides. An attractive hypothesis is that this modulation may occur through activation of the *rut*-encoded cyclase via an uncharacterized AMN receptor. Other signaling components illustrated include the protein NF1, which in *Drosophila* is involved in adenylyl cyclase activation. The *dnc*-encoded cAMP phosphodiesterase, the catalytic and regulatory subunits of protein kinase A (C/RI/RII), and a 14-3-3 protein encoded by *leo* are all preferentially expressed in mushroom body neurons. Cell adhesion molecules involved in *Drosophila* odor learning include an integrin encoded by *Vol* and Fasciclin II (Fas II). These molecules are also preferentially expressed in mushroom body neurons. Memory involves at least two temporal components—short- and long-term—which rely upon posttranslational modifications and alterations in gene expression (the latter partly through CREB), respectively.

is expressed in DPM neurons that innervate the axons and axon terminals of mushroom body neurons. The DPM neurons release a modulatory neuropeptide in adults that alters the physiology of mushroom body neurons to help stabilize or consolidate odor memories. Since AMN is similar to vertebrate peptides that stimulate adenylyl cyclase activity and the *rut*-encoded adenylyl cyclase is found primarily in the axon tracts of mushroom body neurons, the physiological effects of the AMN released peptides may be mediated, in part,

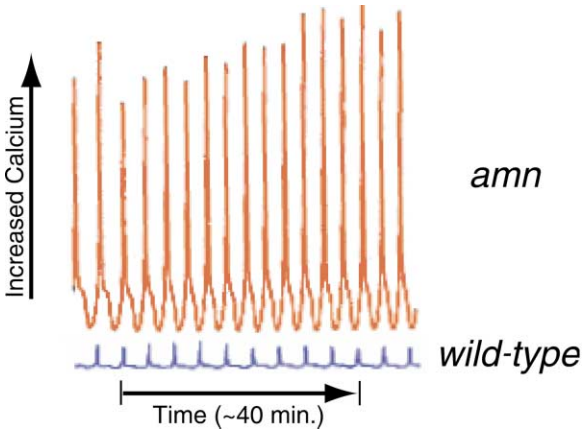

Figure 3. Calcium Oscillations in Mushroom Body Neurons of *amn* and Wild-Type Animals

Spontaneous calcium oscillations are observed in the mushroom bodies of wild-type brains (blue trace) with a periodicity of about 1 every 4 min. Oscillations with the same periodicity are also observed in *amn* mutant brains (red trace) but with an increased magnitude.

through an unknown AMN receptor coupled to this cyclase (Figure 2).

Calcium Oscillations in Mushroom Bodies and Memory Consolidation

What physiological changes, other than a stimulation of adenylyl cyclase and cAMP levels, might be induced in mushroom body neurons by AMN neuropeptides that could correlate with memory stability or consolidation? Kaiser and colleagues now report that calcium levels in mushroom body neurons oscillate, and the magnitude of these oscillations is increased in *amn* mutants (Figure 3; Rosay et al., 2001). They used a novel approach to make these and several other conclusions. A luminescent protein that is sensitive to calcium, aequorin, was expressed in the mushroom bodies of transgenic animals, and the required aequorin cofactor, coelenterazine, was added to dissected brains kept in culture or in some cases, through the head cuticle directly in order to monitor luminescence in the living animal. By following the luminescence over time of brains dissected from normal and mutant animals, and also in response to agonists and antagonists of various neurotransmitter receptors and ion channels, the researchers made several interesting observations.

First, the oscillations reflect synchronized increases and decreases of calcium in a large population of mushroom body neurons, since the sensitivity and resolution of the method is too low to detect changes in one or a few mushroom body neurons. The synchronization must be established by some internal clock, a network of communication among mushroom body neurons, and/or perhaps by some coherent external input. Second, the oscillations are spontaneous; that is, they were observed without any electrical or chemical stimulation of neurons that synapse with mushroom body neurons. Third, the calcium levels oscillate with a mean period of about 4 min, although there is significant variation in the period and the amplitude from brain-to-brain. This spontaneous periodicity is much different than the oscillation in local field potentials of mushroom body neurons

previously discovered by Gilles Laurent and colleagues (Laurent et al., 1998). The Laurent oscillations occur 7000 times faster than the Ca^{2+} oscillations and are driven by inputs from antennal lobe relay neurons in response to odorants. The two types of oscillations are, therefore, unlikely to be related. Fourth, the oscillations emerged late during development, appearing about 2 days after pupariation. This could reflect a maturation of the mushroom bodies neurons at this time point or perhaps the birth of new mushroom body neurons with oscillatory potential. An attractive possibility is that α/β mushroom body neurons trigger the oscillations or are themselves responsible for the oscillations. This class of mushroom body neurons is born beginning at pupariation, while α'/β' and γ mushroom body neurons are born during larval stages (Lee et al., 1999). Fifth, the oscillations require extracellular calcium and were blocked by inhibitors of voltage-gated calcium channels such as verapamil or diltiazem but were unaffected by thapsigargin, an agent that disrupts the internal stores of calcium. This indicates that the oscillations observed may initiate from influxes of calcium through plasma membrane channels. Sixth, a variety of pharmacological agents that interact with ion channels and neurotransmitter receptors disrupted the calcium oscillations. These include inhibitors of voltage-gated sodium channels, potassium channels, acetylcholine receptors, and GABA receptors. Selective activators of these components also blocked the oscillations, including acetylcholine receptor and GABA receptor agonists. In general, the effect of these compounds was to eliminate or reduce the amplitude of the oscillations, although GABA receptor pharmacocompounds notably affected both amplitude and periodicity. It is impossible to draw a plausible biophysical model for the oscillations from these combined pharmacological interventions. The deepest conclusion that can be made is simply that the oscillations require electrical activity, either from within the mushroom body neurons themselves or within neurons that provide input to the mushroom body neurons. Finally, the amplitude but not the periodicity of the oscillations was increased in *amn* mutant brains (Figure 3). This increase was well above the normal variation in amplitude observed in brains from wild-type animals. From preliminary experiments, Rosay et al. (2001) report no changes in the oscillations in three other learning mutants examined.

Many outstanding questions are raised by these observations. For instance, in which cellular compartment of the mushroom bodies do calcium levels rise and fall—the axons, dendrites, or cell bodies? This is critical to know in order to make an educated guess of the biological significance of the oscillations. If the oscillations occur principally in the cell bodies, then one might invoke models of how the calcium affects transcription through calcium-sensitive transcription factors. If it occurs principally in the dendrites, then one might guess that calcium-sensitive enzymes localized postsynaptically, such as calcium-dependent protein kinase II, might be a principal target of the calcium alterations. From a figure published by Rosay et al. (Figure 2, right panel), one would guess that the oscillations detected are within the mushroom body cell bodies and perhaps the dendrites, although this is difficult to discern.

How are the oscillations produced and how are they modulated? Amplitude modulation may occur through the released AMN neuropeptides impinging upon mushroom body axons, a neurohormonal effect, or by some other indirect mechanism. If amplitude modulations are mediated through an AMN-receptor coupled to the *rut*-encoded adenylyl cyclase as depicted in Figure 2, then one would expect that the calcium oscillations would also be elevated in amplitude in *rut* mutants. Rosay et al. (2001) report that preliminary experiments have failed to show this. Another issue is whether the oscillations are specific to one type of mushroom body neuron, or whether they are a property of all types of mushroom body neurons.

The most important unknown, of course, is the biological significance of the oscillations to mushroom body cell physiology and to their role in odor learning and memory. One interpretation offered by Rosay et al. (2001) is that oscillation amplitude may in some way be responsible for the consolidation of odor memories. Although this is possible, there is no direct support for this idea. The oscillations are, nonetheless, a new and distinguishing physiological feature of mushroom body neurons that may be relevant to memory. In addition, DPM neurons—a site of *amn* expression and potential modulators of oscillations—must also now be placed with mushroom body neurons as those with dominion over olfactory memories in insects.

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